ELSEVIER Chemico-Biological Interactions 119–120 (1999) 463–470

Chemico-Biological Interactions

Organophosphate skin decontamination using immobilized enzymes

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Abstract

We previously demonstrated that a combination of cholinesterase (ChE) pre-treatment with an oxime is an effective measure against soman and sarin. We describe here a novel approach for the preparation of covalently linked ChEs which are immobilized to a polyurethane matrix. Such preparation of ChE-sponges enhances the stability and usefulness of the enzymes in non-physiological environments. The ChE-sponges, which can be molded to any form, can effectively be used to remove and decontaminate organophosphates (OPs) from surfaces, biological (skin or wounds) or otherwise (clothing or sensitive medical equipment), or the environment. The ChE-sponges retained their catalytic activity under conditions of temperature, time, and drying where the native soluble enzyme would rapidly denature, and can be reused in conjunction with oximes many times. The ChE-sponge in the presence of oxime repeatedly detoxified OPs such as DFP or MEPQ. These developments in ChE technology have extended the applicability of OP scavengers from in vivo protection, to a variety of external detoxification and decontamination schemes. In addition to treatment of

Abbreviations: AChE, acetylcholinesterase; ChE, cholinesterase; OP, organophosphorus; BChE, butyrylcholinesterase; PUF, polyurethane foam sponge; HI-6, *N,N'*-(oxydimethylene)(pyridine-4-carboxyamide)(pyridine-2-aldoxime); DFP, diisopropyl fluorophosphate; TMB4, *N,N'*-trimethylene bis(pyridine-4-aldoxime); MEPQ, 7-(methylethoxyphosphinyloxy)-1-methylquinolinium iodide; sarin, *O*-isopropyl methylphosphonofluoridate.

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0009-2797/99/$ - see front matter Published by Elsevier Science Ireland Ltd.
PII: S0009-2797(99)00059-9

OP-contaminated soldiers, the ChE-sponge could protect medical personnel from secondary contamination while attending chemical casualties, and civilians exposed to pesticides or highly toxic nerve agents such as sarin. Published by Elsevier Science Ireland Ltd.

Keywords: Acetylcholinesterase; Cholinesterases; Organophosphorus; Bioscavenger; Oxime reactivation; Polyurethane foam sponge; Decontamination

1. Introduction

Organophosphorus compounds (OPs) are potent inhibitors of acetylcholinesterase (AChE, EC 3.1.1.7) and butyrylcholinesterase (BChE, EC 3.1.1.8) because they bind specifically and in most cases irreversibly to the active-site serine, yielding an inactivated phosphorylated enzyme. The sequel of OP toxicity is a cholinergic crisis in man. We have described an antidotal therapy using cholinesterases to detoxify OP compounds: the enzyme bioscavenger approach. This procedure was effective against a variety of OP compounds since the added enzymes are the same targets of the chemical toxins [1–3]. While the use of cholinesterase (ChE) as a single pretreatment drug for OP toxicity provided complete protection, a stoichiometric amount of enzyme was required to neutralize the OP in vivo. To increase the OP:enzyme stoichiometry, we combined enzyme pretreatment with the oxime HI-6 so that the catalytic activity of OP-inhibited AChE is rapidly and continuously restored (the OP is continuously detoxified) before aging of the enzyme-OP complex can occur.

In addition to the in vivo antidotal therapy, the in vitro reactivation of OP-inhibited ChEs by oximes has important applications for medical, surgical, and skin decontamination, and also the decontamination of materials, equipment, and the environment. The currently accepted methods for decontamination of large areas, personnel, and materials use sodium hydroxide and bleach treatment, which pose environmental consequences and are harmful. We will describe the development of a sponge product, composed of a ChE, oxime, and polyurethane foam combinations for the removal and decontamination of OP compounds from biological surfaces such as skin.

2. Results and discussion

2.1. Polyurethane foam (sponge) synthesis

For the ChEs, we envisioned a reusable decontamination sponge. Therefore, we chose a porous polyurethane foam formed in-situ from water-miscible hydrophilic urethane prepolymers and the enzyme [4–7]. The polyurethane foam (PUF) investigated in this study was the polyether prepolymer derived from TDI, tolyl diiso-

cyanate (Hypol prepolymer TDI 3000). The enzymes can attach to the inert foam at multiple points dependent upon the available free aliphatic amines (lysine and arginine residues) available on their surface. Based on molecular modeling (Fig. 1), there are at least 1 Lys and 29 Arg water accessible residues on the surface of fetal bovine serum (FBS)-AChE to couple to the PUF, while 26 Lys and 26 Arg residues were modeled for Equine-BChE (truncated after residue 537). The majority of the Lys and Arg residues were found on the backside of the ChEs, although a few are close to the rim of the gorge (active site opening). BChE shows significantly more Lys residues located on the front of the enzyme than AChE. As shown in Fig. 1, the active site remains open, thereby permitting substrate and inhibitors access to the catalytic triad. Remarkably, after only vigorous mixing at ambient temperature, the sponge cures in less than 30 min, molded to the shape of its container, as shown in Fig. 2. Therefore, the ChE-sponges could be formed into towelettes to decontaminate skin or 4×4 inch surgical pads to prevent cross-contamination of medical personnel, or even mops to decontaminate sensitive equipment.

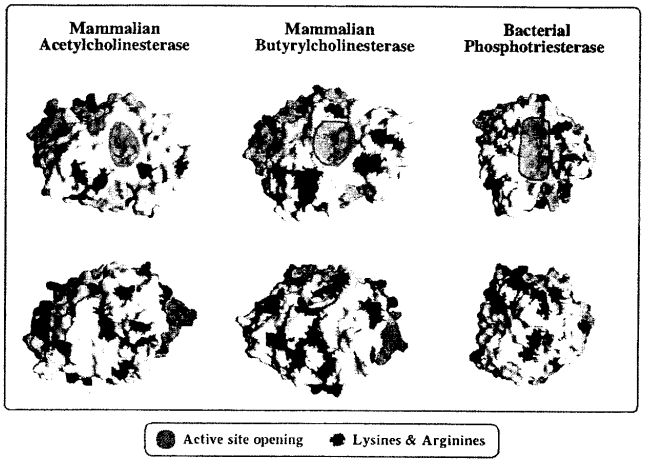

Fig. 1. Modeled surfaces of ChEs and triesterase. The top row represents a view of the front of the enzymes with the lip of the active site gorge shaded in the center. The bottom row depicts the back side of the enzymes (180° rotation). The lysine and arginine residues on the surface, which are potential coupling sites to the polymer, are shaded dark in both the top and bottom row. Modeling of the enzymes was performed on a Silicon Graphics workstation using molecular modeling software (Insight II, Biosym Technologies, San Diego, CA).

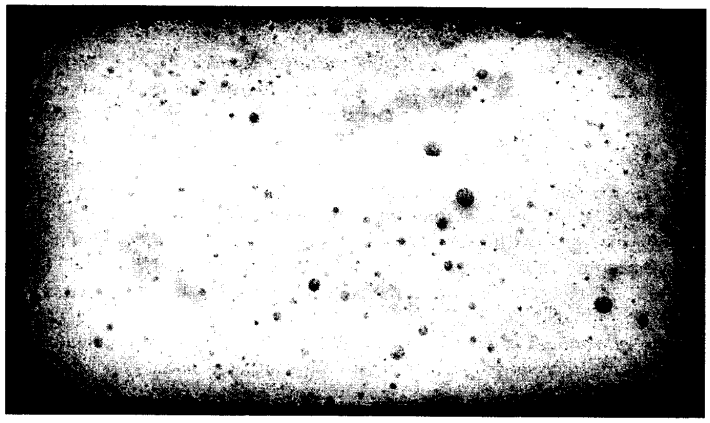

Fig. 2. FBS-AChE homogeneously immobilized to the polyurethane foam (sponge). Hypol prepolymer TDI 3000 was purchased from Hampshire Chemical, Lexington, MA and Pluronic P-65 surfactant from BASF Specialty Chemicals, Parsippany, NJ. A typical synthesis of the sponge consists of enzyme in phosphate buffer containing 1% (final concentration) surfactant and 6 g of prepolymer. The 2-phase system was mixed at 2500 rpm for 50 s and injected with a syringe into a suitable mold. The sponge containing the covalently coupled ChEs cured in less than 30 min, molded to the shape of its container.

2.2. Characteristics of immobilized ChEs

2.2.1. Sponge capacity

Our results demonstrate the following characteristics of sponges containing either immobilized AChE or BChE. As expected for a uniform immobilization of AChE or BChE throughout the sponge, a linear correlation was established between the weight of the TDI sponge and enzyme activity. We found that the TDI sponge has a significantly higher loading capacity for ChEs than the amount of purified AChE we added. When increasing amounts of non-specific protein (bovine serum albumin, BSA) were added to a constant amount of purified AChE and the mixture cured, there was no reduction in sponge ChE activity, even when there was a 1000-fold excess of BSA. These results suggest that even higher potency sponges can be synthesized from purified ChEs or by synthesizing with additional or other ChEs, thereby substantially increasing the effectiveness of the preparation.

2.2.2. Immobilized enzyme stability

When either AChE or BChE TDI PUF was exhaustively washed and activity determined, and the wash and assay cycle repeated more than twenty times over three days, no decrease in activity occurred, indicating that the sponges could be used repeatedly. These results also demonstrate that the ChEs were covalently cross-linked in the sponge matrix and that the ChEs would not leach out to skin or equipment. While long-term temperature and stability of the ChE TDI sponges are

Table 1
Equilibrium and kinetic constants for soluble and immobilized ChEs

Enzyme	Form	K_m	K_{cat}
FBS-AChE	Soluble	0.119 ± 0.010	2.8×10^5
	Immobilized	1.09 ± 0.10	5.9×10^4
Equine-BChE	Soluble	0.127 ± 0.020	3.1×10^4
	Immobilized	1.20 ± 0.18	1.8×10^4

still under evaluation, we have found that ChE activity remains unchanged at 4°C after more than 3 years and at 25°C after about eight months, or at 45°C after 7 months (the length of the current tests). The TDI sponge imparts remarkable stability to the ChE-sponges; up to about 50% of the original ChE-sponge activity activity remained after 16 h at 75°C, conditions under which soluble enzyme would exhibit no activity. The ChE-sponges can be exhaustively dried under vacuum at 22°C and then rehydrated without loss of the enzyme activity.

2.2.3. Immobilized and soluble ChEs' kinetic and inhibition constants

An initial rates method was used to determine the parameters K_m and k_{cat} for immobilized and soluble AChE and BChE. The number of active sites of either the coupled or soluble ChEs was determined by titration with MEPQ (7-(methylethoxyphosphinyloxy)-1-methylquinolinium iodide). As shown in Table 1, the K_m values for the AChE-PUF was about 10-fold greater than for the corresponding soluble enzyme, while k_{cat} values were less dramatically affected. In contrast to the change in K_m values for the immobilized ChEs, there was no observed shift in the pH profile for soluble or immobilized enzymes. More important, the bimolecular rate constants for the inhibition of AChE-PUF and BChE-PUF and the corresponding soluble enzymes by MEPQ at 25°C, reported in Table 2, showed no significant difference between soluble and covalently bound enzymes. Thus, the OP interacts similarly with soluble and immobilized ChEs.

2.2.4. Organophosphate inhibition and oxime reactivation of immobilized AChE

We evaluated the AChE-sponge for inhibition by OPs such as DFP or MEPQ, and subsequent reactivation by oximes. The activity of AChE-sponge could be almost completely inhibited by OP in the presence of 2 mM HI-6 only at an

Table 2
Time-dependent inhibition of ChEs (soluble and immobilized) by the organophosphate MEPQ

ChE	Form	Bimolecular rate constant (M^{-1} min^{-1}) ± S.D.
FBS-AChE	Soluble	$1.59 \pm 0.52 \times 10^8$
	Immobilized	$1.00 \pm 0.28 \times 10^8$
Equine-BChE	Soluble	$4.15 \pm 0.78 \times 10^7$
	Immobilized	$4.21 \pm 2.00 \times 10^7$

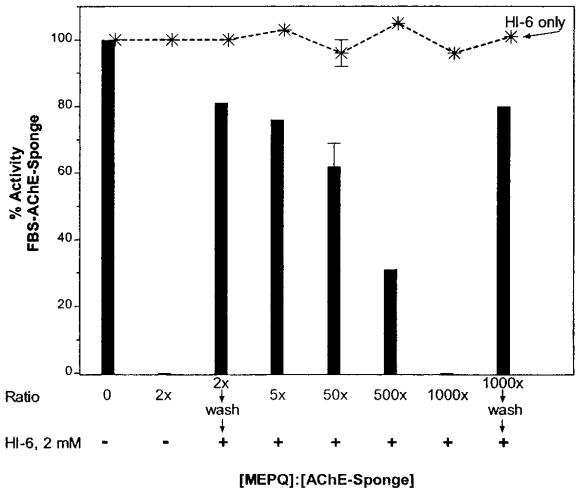

Fig. 3. ChE-PUF were incubated with increasing concentrations of OP with or without 2 mM oxime reactivator, and the remaining ChE activity in the sponge was determined after rinsing the sponges in phosphate buffer to remove unreacted OP and oxime. To test for reactivation of OP-inhibited ChE-PUF, oxime was added to washed and inhibited PUFs. Note that even after addition of 500-fold molar excess of OP to the AChE sponge, some enzyme was uninhibited in the presence of HI-6, and that after almost complete inhibition (1000x), the sponge could be rinsed with HI-6 and its activity restored for subsequent use.

[OP]:[AChE-sponge] of 1000 (ratio of the concentration of OP to active sites, Fig. 3). However, simply rinsing the sponge with HI-6 buffer in the absence of OP restored most of the original cholinesterase activity, permitting the AChE-sponge to be cycled over and over, at least twenty times. Furthermore, the polyurethane matrix retains its sponge-like characteristics during the cycling. Similarly, OP-inhibited BChE-sponge could be reactivated by TMB4, the more effective oxime for BChE reactivation. Thus, we show the feasibility of using ChE-sponges to detoxify more than the stoichiometric amount of OPs.

The effectiveness of ChE-immobilized sponges for detoxification of OPs is amplified by oxime reactivators in the in vitro environment. Since OPs in a diluted aqueous media are more readily absorbed by the skin, the use of the sponge for skin decontamination is the preferred mode: the PUF absorbs the OPs, preventing further penetration through the skin, and the immobilized ChEs detoxify the OPs. In addition, immobilized enzyme-sponges will not cause any immune reactivity when used on wounds or skin, since these enzymes do not leach out as observed in encapsulated preparations such as liposomes or cyclodextrans.

3. Conclusions

We have demonstrated the rapid in situ copolymerization of ChEs and PUF at room temperature, and showed that the ChE-sponges exhibit high enzyme activity, making them suitable for a wide variety of decontamination processes. These TDI ChE-sponges exhibit remarkable long-term stability and increased resistance to elevated temperatures. The immobilized enzymes show similar activity to and the unique properties of their soluble but unbound form. Due to the large capacity of the prepolymer for protein, high activity sponges can be synthesized from purified ChEs, substantially increasing their efficacy. Multiple OP-hydrolyzing enzymes can be co-immobilized on one sponge, including phosphotriesterases (paraoxonase or OP hydrolases) and/or cholinesterases. The advantage of including OP hydrolases in the multi-enzyme component is that they detoxify all phosphonylated oximes with little substrate specificity.

In addition to decontamination of skin, wounds, and personnel, the enzyme-sponges can be utilized for preventing cross-contamination of medical and clinical personnel. Still more uses for these formulations could include decontamination foams as masks and in garments, replacing carbon filters that absorb OPs without inactivating them. The ChE-PUF could be used in chemical-biological sensors as electrochemical OP probes. OPs in the environment could be contained and decontaminated if the ChE-PUF were incorporated into firefighting foams. The enzyme-foams could be used to decontaminate sensitive equipment without posing new environmental disposal problems, since the final products are rendered inert. Indeed, the sponge should be suitable for a variety of detoxification and decontamination schemes for both chemical weapons and civilians exposed to pesticides or highly toxic OPs such as sarin [8].

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